EXHIBIT B

PASTEUR MÉRIEUX
Sérums & Vaccins
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Recherche - Formulation

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Cher Farrukh,

Je t'envoie ci-joint un rapport sur les propriétés adjuvantes d'un lipide cationique fusogène, le DC-Chol. Comme ce domène est très compétitif M. André Bourgouin et Pierre Meulien m'ont demandé d'envoyer ces résultats préliminaires sur le sol américain dans les meilleurs délais.

Il faudrait que tu accuses réception de ce document en remplissant la rubrique prévue à cet effet (page 4 du rapport) et que tu l'archives soigneusement.

Au fur et à mesure de l'avancement de nos travaux, je te transmettrai des rapports de plus en plus détaillés.

Avec tous mes remerciements,

Très cordialement.

Jean Haensler.

cc/ Pierre Meulien, André Bourgouin.

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Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers

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Abstract

DNA-based vaccines are considered to be potentially revolutionary due to their ease of production, low cost, long shelf life, lack of requirement for a cold chain and ability to induce good T-cell responses. Twenty healthy adult volunteers were enrolled in a Phase I safety and tolerability clinical study of a DNA vaccine encoding a malaria antigen. Volunteers received 3 intramuscular injections of one of four different dosages (20, 100, 500 and 2500 μg) of the *Plasmodium falciparum* circumsporozoite protein (*Pf*CSP) plasmid DNA at monthly intervals and were followed for up to twelve months. Local reactogenicity and systemic symptoms were few and mild. There were no severe or serious adverse events, clinically significant biochemical or hematologic changes, or detectable anti-dsDNA antibodies. Despite induction of excellent CTL responses, intramuscular DNA vaccination via needle injection failed to induce detectable antigen-specific antibodies in any of the volunteers. Published by Elsevier Science Ltd.

Keywords: DNA vaccine; Malaria; Clinical trial

It has been 9 yr since the first report that intramuscular administration (IM) of "naked" plasmid DNA resulted in expression by myocytes of the protein encoded by the DNA [1], 7 yr since the first report of the immunogenicity in mice of a plasmid DNA vaccine [2], more than 5 yr since IM administration of plasmid DNA was reported to protect mice against influenza [3] and malaria [4]. In the intervening years there has been an explosion of interest in DNA vaccines with reports of their efficacy both in small and large animals for preventing and treating many infectious diseases, cancer and autoimmune diseases [5].

The enthusiasm for DNA vaccines is based on their demonstrated ability to be immunogenic and to provide protective efficacy in animals. In addition, DNA vaccines are easy to produce and purify [6] have a relative low cost, long shelf life and may not require a

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cold chain. In considering single formulations of vaccines effective against many infectious agents, vaccines against complex multi-stage microorganisms like *Plasmodium falciparum*, rapid response vaccines against newly emerging pathogens and "individualized" vaccines for cancer patients, DNA based vaccines are considered to be potentially revolutionary [7].

When expression of protein in myocytes and immunogenicity of DNA vaccines in experimental animals were first reported, skeptics considered the findings to be artifactual. When reproducible scientific experiments established the validity of these findings, many believed that it would be impossible to conduct human studies because such vaccines would be inherently unsafe. One major concern was that after injection the DNA would integrate into the recipient host's chromosome leading to mutagenesis and potentially insertional carcinogenesis. Therefore, human studies could be initiated only if this concern was greatly diminished. Animal studies involving plasmid DNA injections have shown that any mutations from a potential integration event would be infrequent and result in a calculated mutation rate much lower than the spontaneous mutation rate for mammalian genomes [8,9]. A second concern was that immunization with plasmid DNA would induce anti-DNA antibodies and this would accelerate the development of autoimmune diseases. Animal studies have shown non-significant increases in anti-DNA antibodies and no evidence of autoimmune disease induction or acceleration after administration of plasmid DNA injections [10].

The technology of DNA vaccines appears to be ideally suited to the development of a multi-gene malaria DNA vaccine which can effectively target multiple stages of the *P. falciparum* life cycle [11]. Our initial focus has been on the development of a vaccine which induces protective $CD8^+$ T cell responses directed at the infected hepatocyte, since $CD8^+$ T cells have been implicated as critical effector cells in pre-erythrocytic stage protection in rodent models (reviewed in [12,13]). Our strategy for development of a multi-gene malaria DNA vaccine has included assessment of in vitro expression and in vivo immunogenicity of plasmid DNA in rodents and nonhuman primates before transitioning to clinical studies in humans [13,14]. Accordingly, a *P. falciparum* circumsporozoite protein (*Pf*CSP) expressing plasmid was evaluated for antigen expression in vitro by immunoblot analysis of cultured mammalian cells following transient transfection and for the capacity to induce antigen-specific antibody and cytotoxic T lymphocyte (CTL) responses in mice [15] and nonhuman primates [16]. Studies in the rodent model (reviewed in [17]) demonstrated induction of antigen-specific CTL and antibody responses following immunization with plasmid DNA encoding antigens expressed in Plasmodium-infected hepatocytes. Studies further established that the IM route of immunization was optimal for induction of $CD8^+$ Th1 immune responses, as reported in other systems [18]. Subsequent studies demonstrated that all of six Rhesus monkeys immunized IM with the *Pf*CSP plasmid, either alone or in combination with up to four plasmids encoding other pre-erythrocytic liver stage *P. falciparum* proteins, had detectable antigen-specific CTL and/or antibody responses [16].

These studies in rodent and non-human primate models provided the foundation for Phase I clinical evaluation of a vaccine designed to induce $CD8^+$ Th1 immune responses directed against the infected hepatocyte in humans, using the IM route of administration. The *Pf*CSP plasmid was subsequently produced under current Good Manufacturing Practices conditions (cGMP) and was extensively characterized for safety in mice and rabbits [19] prior to clinical testing in humans.

Two previously reported clinical trials of DNA vaccines were conducted in subjects with HIV infections [20,21]. In this report, we demonstrate for the first time the safety and tolerability of a DNA vaccine in healthy humans who have been followed for up to 12 months after vaccination. We have previously reported the induction of antigen-specific, MHC-restricted $CD8^+$ CTL in 11 of the 20 volunteers from all DNA dosage groups [22]. Here, we report that measurable antigen-specific antibody responses were not detected in any of the 20 study volunteers suggesting that the IM route of administration may not be optimal for the induction of antibodies by a DNA vaccine in human subjects.

1. Materials and methods

1.1. Volunteers

Twenty healthy, malaria-naive, adult volunteers from the communities surrounding the participating facilities were recruited by noncoercive means under a protocol approved by the Institutional Review Boards of the participating facilities. Potential and theoretical risks associated with receiving the *Pf*CSP investigational vaccine were discussed with the volunteers. The research protocol for human participants in this study was approved by the Naval Medical Research Center's Committee for the Protection of Human Subjects, the U.S. Army Medical Research Institute of Infectious Diseases Human Use Committee and the Surgeon General's Human Subjects Research Review Board, in accordance with the U.S. Navy regulation (SECNAVINST 3900.39B) governing the use of human subjects in medical research. Written informed consent was obtained from all volunteers. It was made very clear to

the volunteers that they were free to withdraw from the study at any time without penalty or loss of any benefits.

All 20 study subjects underwent a screening evaluation consisting of a medical history, physical examination, complete blood counts, clinical biochemistries, urinalysis, complete class I and II HLA typing, antinuclear antibody (ANA) (ELISA, Helix Diagnostics, West Sacramento, CA) and anti-dsDNA antibody (ELISA, Diamedix Corporation, Miami, FL) tests and serologic studies for previous exposure to or infection with HIV, hepatitis B, hepatitis C, PfCSP or vaccinia. Volunteers were excluded if they had HIV infection, active hepatitis B or C, anemia, cardiac, hepatic, renal, or autoimmune disease, pre-existing anti-dsDNA antibodies or positive ANA ELISA, pre-existing *Pf*CSP antibodies, allergy to any study vaccine component, splenectomy, known immunodeficiency, or any findings which in the opinion of the Principal Investigator (PI) would increase the risk of having an adverse outcome from participation. In addition, all female volunteers were screened for serum beta-HCG immediately before each vaccination and excluded if this was positive.

1.2. Vaccine

The study vaccine, VCL-2510 (Fig. 1), is a closed circular DNA plasmid produced under cGMP from bacterial cells grown in kanamycin selective media [6]. VCL-2510 plasmid, which has been previously described [15], contains the full length gene encoding *Pf*CSP, a protein antigen found on the surface of *P. falciparum* sporozoites and within infected hepatocytes. Expression of *Pf*CSP is controlled by the promoter/enhancer of the human cytomegalovirus immediate early (CMV IE) gene, the 5′ untranslated region of the CMV IE gene (including intron A) and the transcriptional terminator from the bovine growth hormone gene [23]. The *Pf*CSP coding sequence is modified at the 5′ end by addition of the sequence encoding the leader peptide from human tissue plasminogen activator protein (hTPA) to enhance expression and secretion of the antigen in mammalian cells [15]. The plasmid contains two open reading frame sequences. One encodes the kanamycin resistance protein which is expressed in bacterial cells and the other encodes hTPA leader/*Pf*CSP fusion protein which is expressed in mammalian cells [15]. There are no known viral or oncogenic protein coding sequences in the VCL-2510 plasmid. Extensive preclinical safety studies with VCL-2510 in experimental animals were conducted prior to the clinical trial [9,19].

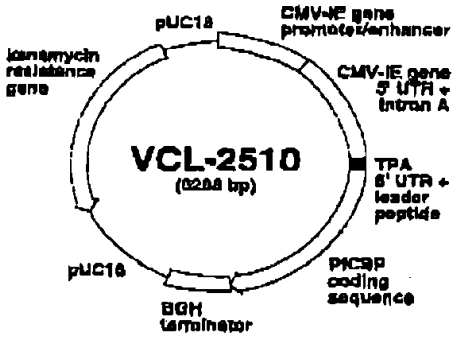

Fig. 1. Diagrammatic representation of the DNA vaccine plasmid. Plasmid map of the vaccine VCL-2510 indicating the relative positions of the human cytomegalovirus immediate early (CMV-IE) gene promoter/enhancer and the CMV-IE gene 5′ untranslated region (UTR) plus intron A, the sequence encoding the leader peptide of human tissue plasminogen activator protein (TPA) fused to the 5′ end of the sequence encoding the *P. falciparum* circumsporozoite protein (*Pf*CSP) followed by the transcription termination region of the bovine growth hormone (BGH) gene and the bacterial gene encoding kanamycin resistance all of which comprise this circular bacterial plasmid (pUC18) derivative. The two protein encoding regions of VCL-2510 are shown as arrows indicating their orientation of transcription.

1.3. Study design

This was an open-label, dose-escalating Phase I safety and immunogenicity trial of the *Pf*CSP DNA vaccine in healthy adult volunteers. The 4 dose groups were: 20, 100, 500 and 2500 μg. The group assignment was made in such a way that the sex, race and HLA background were equally distributed in the 4 groups. Frozen vials of vaccine were thawed at room temperature for 30 min prior to injection. Vaccine was administered as an IM needle injection of 1.0 ml in the deltoid muscle. In each dose group, volunteers received three total injections of the same dose of the vaccine at 0, 4 and 8 weeks, alternating arms between each injection. There was a two-week stagger between the dose groups, with the lowest dose group vaccinated first, to allow for assessment of safety and tolerability before proceeding to the next higher dose.

1.4. Assessment of safety

Volunteers were observed for 30 min after each immunization and again at 1, 2, 7 and 13 days. At each of the follow-ups, directed history and physical examination, including vital signs were done. Blood was obtained for complete blood count and the following serum biochemistries: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (alk phos), bilirubin (bili), creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and urine obtained for urinalysis on the day of immunization, day 1, 7 and 13. At 2 and 6 weeks after the last immunization, blood was obtained for complete blood

count, serum biochemistries and ANA and anti-dsDNA antibodies. In addition, at the 6- and 12-month follow-up visits, blood was also obtained for ANA and anti-dsDNA antibody determinations. All symptoms, signs and laboratory abnormalities were considered adverse events and classified using a standard scale (see Table 1).

1.5. Assessment of antibody response in volunteers

Plasma samples obtained 2 and 4 weeks after both the second and third immunization and also 6 and 14 weeks after the third immunization were assessed for anti-CSP antibodies against recombinant *Pf*CSP protein and against synthetic peptides of the B-cell epitope from the central repeat region of the *Pf*CSP by ELISA and against intact sporozoites by IFAT. Recombinant *Pf*CSP was derived from a genetic construct of *E. coli* in which it was expressed from a plasmid as a histidine-tagged (His_6-tagged), intracellular protein. The plasmid was constructed as follows. The full-length *Pf*CSP gene encoding amino acids M_1 to N_{397} (single letter amino acid sequence code) was PCR-amplified from genomic DNA of the 3D7 strain of *P. falciparum*. The following primers were used for PCR-amplification: sense oligonucleotide 5′-GCCCATGGTTATGATGAGAAAATTAGCTATT-3′ and antisense oligonucleotide 5′-GCGGATCCAATAAGGAACAAGAAGGATAA-3′. The PCR-amplified DNA fragment was digested with restriction endonucleases NcoI and BamHI then ligated into NcoI/BamHI predigested pQE60 plasmid DNA (Qiagen, Chatsworth, CA), an expression plasmid that adds six histidine amino acid residues to the carboxy-terminus of the expressed protein. Following ligation, selection of clones and characterization by restriction analysis, the plasmid designated pb*Pf*CSP was isolated for production of recombinant *Pf*CSP. *E. coli* M15 containing pbPfCSP was used for production of recombinant protein. A 300 liter fermentor (New Brunswick Scientific, Edison, NJ) at the Department of Biologics Research, Walter Reed Army Institute of Research was used to produce a 500 gram bacterial pellet. The bacteria were lysed using a microfluidizer (Microfluidics, MD) on ice under 15,000 psi air-pressure. The lysed bacteria were centrifuged at 10,000 *g* and the supernatant was filtered using a 0.45 micron filter membrane. From this soluble material the His_6-tagged recombinant protein was bound to a Ni-NTA (Qiagen) chromatography column and protein was eluted from the resin with 0.2 M sodium acetate, pH 4.5. The protein was further purified by Superdex-75 (Pharmacia, Uppsala, Sweden) column chromatography. The amino acid sequence of the NH_2-terminus of the purified protein was determined by automated Edman degradation (Biological Resources Branch, National Institute of Allergy and Infectious Diseases). This analysis showed that most of the purified protein had sustained a cleavage event during synthesis that removed the first 93 amino acid residues. Thus, the bulk of purified protein in the preparation lacked the amino terminus of the full-length *Pf*CSP protein and initiated at L_{94}. The synthetic peptide $(NANP)_{10}$ was described previously [24].

Table 1
Adverse Events: incidence, severity and relationship to vaccine administration

Relationship	Severity			
	mild[a]	moderate[b]	severe[c]	serious[d]
None	19	1	0	0
Unlikely	37	2	0	0
Possibly	24	1	0	0
Probably	6	0	0	0
Definitely	21	0	0	0

[a] Mild - symptoms can be ignored.
[b] Moderate - Symptoms minimally affect activity or are relieved by medications.
[c] Severe - symptoms prevent performance of activities of daily living or persist despite medications.
[d] Serious - symptoms are life/limb threatening, are permanently disabling, require inpatient hospitalization, or result in death.

Enzyme-linked immunosorbent assay (ELISA) was performed as previously described [25] with slight modifications. Briefly, 50 μl of recombinant *Pf*CSP protein (0.25 μg/ml) or synthetic peptide $(NANP)_{10}$ diluted in PBS was added into wells of Immunolon II ELISA plates (Dynatech Laboratory Inc., Chantilly, VA). Plates were incubated for 6 h at ambient temperature. The wells were washed 3 times with PBS containing 0.05% Tween 20 (washing buffer) and incubated overnight at 4°C with 100 μl of 5% nonfat dry milk in PBS (blocking buffer) per well. After washing 3 times, the wells were incubated for 2 h with 50 μl of two-fold serial dilutions of test plasma and control human plasma diluted in 3% nonfat dry milk in PBS (diluting buffer). The wells were washed 3 times, incubated for 1 h with peroxidase-labeled goat anti-human IgG (H+L) or IgM (μ) (Kirkegaard and Perry, Gaithersburg, MD) diluted 1:4000 in diluting buffer, then washed again 3 times. The wells were incubated for 10 min with 100 μl of a solution containing ABTS (2,2-azino-di-(3-ethyl-benzthia-zoline sulfonate) substrate (Kirkegaard and Perry, Gaithersburg, MD). Color reaction was measured in a micro-ELISA automated reader (Dynatech, MR5000) at OD 410 nm. All reaction steps except blocking were performed at room temperature. The positive control was plasma from a volunteer immunized with *P. falciparum* radiation attenuated sporozoites. Mean ± SD of the OD readings of quadruplicate assays were recorded. Samples were considered positive if the mean OD value of the plasma

sample post-immunization was greater than the mean OD plus 3 standard deviations of the plasma sample pre-immunization.

Indirect fluorescent antibody test (IFAT) was performed as previously described [26] with a slight modification. Briefly, antigen slides containing 8000 air-dried *P. falciparum* (NF54) sporozoites per well were incubated in a moist chamber for 30 min at 37°C with two-fold serial dilutions of test and control human plasma diluted in PBS containing 0.2% Evans Blue (diluting buffer). The slides were washed 3 times in PBS and further incubated for 30 min at 37°C with FITC-labeled goat anti-human IgG (H + L) (Kirkegaard and Perry, Gaithersburg, MD) diluted 1:50 in diluting buffer. The slides were washed again, mounted in a Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA) and examined under an Olympus UV microscope. Positive control was a plasma sample from a volunteer immunized with radiation attenuated sporozoites of *P. falciparum*.

1.6. Murine antibody studies

Two groups of four female 3- to 5-week-old BALB/c, B10BR and C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) and outbred CD-1 mice (Charles River Laboratory, Wilmington, MA) were immunized with 100 mcg of the cGMP VCL-2510 vaccine at 0, 3 and 6 weeks either intramuscularly in both the tibialis anterior muscles or intradermally in the tail at 3 sites. The mice were bled for sera 10 days after the last dose. Antibodies in sera against intact parasites were determined by IFAT as previously described [26].

2. Results

2.1. Study population

Twenty volunteers were enrolled and assigned to each of 4 dose groups. The median age was 25 with a range of 20 to 28. Eighty percent were male, 75% Caucasians and 85% active duty military personnel. All 5 volunteers in each of groups 1 and 3 completed the study and received the 3 injections at scheduled times. Of the 5 volunteers in group 2, 1 withdrew before receiving the third injection due to unrelated chronic medical and family problems and 1 received the third injection 2 weeks late. In group 4, one volunteer's participation in the study was terminated prior to receiving the third injection due to unexpected pregnancy. All 20 volunteers returned for the 6-month follow-up visit and 19 volunteers returned for the 12-month follow-up visit.

2.2. Safety

All doses of the vaccines were well tolerated in all volunteers. Most of the adverse events were mild (Table 1). The 4 moderate adverse events were as follows: 1 episode of iliac crest pain from unrelated bone marrow donation (20 μg dose group), 1 episode of enterocolitis treated with antibiotic (100 μg dose group), 1 episode of elevated serum CPK from excessive weight lifting (100 μg dose group) and 1 episode of headache 10 days after vaccine injection which resolved with acetaminophen (100 μg dose group). There were no episodes of severe or serious adverse events. The most common complaint, pain and tenderness at the injection site, was mild, lasting less than 48 h and required no medication (Table 2). The injection site pain and tenderness were more common in group 4, the highest dose group. There were no clinically significant serum biochemical abnormalities in any subject throughout the study. There was also no evidence of proteinuria, hematuria, or any other abnormalities in the urinalyses. None of the 20 volunteers had detectable ANA or anti- dsDNA antibodies by screening ELISA at baseline and at 2 and 6 weeks after the last injection. At the 12 month time point, one volunteer (20 μg group) was positive for ANA by screening ELISA and borderline positive (1:40) by confirmatory IFAT. In retrospect, a specimen from that volunteer taken before immunization was also positive by IFAT at 1:40. When tested 3 months later, (15 months after the first immunization), this subject had the same ANA and anti-dsDNA antibody results. None of the volunteers who were evaluated at the 12 month time point (19/20) had detectable anti- dsDNA antibodies (data not shown).

The volunteer who became pregnant during the study was followed throughout her pregnancy. Her pregnancy, labor and delivery were uneventful. There were no abnormalities noted in the infant at birth.

2.3. Anti-PfCSP antibody responses

Anti-CSP antibodies were not detected by ELISA or IFAT in any of the plasma samples collected from the 20 volunteers at multiple time points throughout the study.

2.4. Murine immunogenicity

Since no antibodies were detected in the study volunteers after IM administration of the vaccine, we conducted a murine study comparing IM and intradermal (ID) administration of the cGMP material used in the clinical trial. Results from that study (Table 3) clearly demonstrate that the plasmid induced antibodies in both outbred and inbred strains of mice. Furthermore,

Table 2
Symptoms and signs in 20 volunteers within 48 h after each injection[a]

Symptoms/signs	Group 1			Group 2			Group 3			Group 4		
	Inj 1	Inj 2	Inj 3	Inj 1	Inj 2	Inj 3[b]	Inj 1	Inj 2	Inj 3	Inj 1	Inj 2	Inj 3[b]
Symptoms												
Local pain	2	0	0	0	0	1	1	1	3	5	5	2
Subjective fever	0	0	0	1	0	0	1	0	0	0	0	0
Chills	0	0	0	0	1	0	0	0	0	0	0	0
Malaise	0	0	0	0	1	1	1	0	0	0	0	0
Myalgias	0	0	1	0	0	0	1	0	0	0	0	0
Arthralgias	0	0	0	0	1	0	1	0	1	0	0	0
Headache	0	0	0	0	1	0	2	0	0	0	0	0
Nausea	1	0	0	0	1	0	0	0	0	0	0	0
Vomiting	0	0	0	1	0	0	0	0	0	0	0	0
Diarrhea	1	0	0	0	2	0	0	0	0	0	0	0
Pruritis/rash	0	3	1	1	0	0	0	0	0	1	0	0
Signs												
Fever ($T \geq 38°C$)	0	0	0	0	0	0	0	0	0	0	0	0
Tenderness	2	1	0	0	0	1	0	1	2	4	4	1
Erythema	0	1	0	0	0	0	1	0	0	1	0	0
Warmth	0	0	0	0	0	0	0	0	0	0	0	0
Induration	0	0	0	0	0	0	0	0	0	0	1	1
Decreased ROM	0	0	0	0	0	0	0	0	0	0	0	0
Adenopathy	0	0	0	0	0	0	0	0	0	0	0	0

[a] Results reported as number of volunteers with symptoms/signs. The 3 doses were administered 4 weeks apart.
[b] Only 4 out of 5 volunteers received the 3rd dose.

antibody responses were significantly greater when the plasmid was administered intradermally as compared to intramuscularly as seen in the three inbred strains of mice (Table 3).

3. Discussion

There have been an increasing number of clinical trials utilizing recombinant DNA for gene therapy against multiple malignancies and metabolic diseases [27]. The safety profiles established by these trials, together with supporting animal studies, have laid the foundation for clinical trials with DNA vaccines against infectious pathogens in healthy human volunteers. The US Food and Drug Administration has issued guidance for production and testing of such vaccines ("Points To Consider On Plasmid DNA Vaccines For Preventive Infectious Disease Indications" 1996, http://www.fda.gov/cber/ptc/plasmid.txt). These guidelines outline animal studies that need to be done prior to proceeding to human trials. The preclinical animal studies carried out by a number of groups have shown that direct administration of plasmid DNA is safe and well tolerated. Mice and cynomolgus monkeys that received repeated intravenous injections of VCL-1005, a plasmid DNA vector expressing both the human class I MHC HLA-B7 heavy chain and the beta 2-microglobulin light chain gene formulated in cationic lipids for use in cancer therapy, did not show any

Table 3
IPAT titers[a] against air-dried *P. falciparum* sporozoites in mice immunized with VCL-2510 comparing IM ($n = 4$) and ID ($n = 4$) administration of vaccine

Strain (type)	Mean titer IM (range)	Mean titer ID (range)	p value[b]
CD-1(outbred)	14,720 (1280– > 20,480)	15,680 (2560– > 20,480)	0.941
BALB/c (inbred)	3920 (320–5120)	8960 (5120–10,240)	0.028
B10BR (inbred)	1760 (640–2560)	11,520 (5120–20,480)	0.024
C57BL/6 (inbred)	2640 (0–5120)	8960 (2560–20,480)	0.208
All inbred[c]	2773 (0–5120)	9813 (2560–20,480)	0.001

[a] Reciprocal of the end-point dilution that is positive.
[b] Group means (arithmetic) were compared using an independent samples t-test. The t-tests were performed using SPSS for Windows version 8.0.
[c] BALB/c, B10BR, C57BL/6.

abnormalities in clinical chemistry, hematology, or organ pathology [28]. A similar safety profile was demonstrated in animals injected with VCL-1102, a plasmid DNA encoding human Interleukin 2 [29]. Mice and rabbits that received repeated intramuscular injections of VCL-2510, the *Pf*CSP malaria DNA vaccine used in this study, did not show any abnormalities in clinical chemistry, hematology, or organ pathology [19]. In addition, by using PCR analysis of various tissues at various time points, it was shown that after IM injection, the VCL-2510 plasmid, although detected initially in all of the highly vascularized tissues, was found only in the injected muscle of mice at later time points [19]. Favorable safety profiles established in animals cleared the first hurdle in the progress toward safety studies in humans.

One of the theoretical concerns regarding DNA vaccines is the potential for the injected DNA to integrate into host chromosomal DNA and, depending on the area of integration, induce mutagenesis and insertional carcinogenesis [30]. Safety studies designed to test for possible integration of plasmid DNA can only be conducted in animal models. In a study designed to test integration of an influenza nucleoprotein DNA vaccine in mice, it was reported that no integration could be detected, using a PCR assay demonstrated to have a sensitivity of 1 to 7.5 plasmid copies per 150,000 nuclei. These PCR assays were carried out at 4 and 8 weeks following a single intramuscular injection [8]. Studies with the *Pf*CSP DNA vaccine VCL-2510 likewise showed little to no plasmid association with genomic DNA [9]. In that study, even if the highest level of plasmid DNA found associated with genomic DNA (30 copies per 150,000 mouse genomes) was covalently integrated, and thus resulted in a mutational event, the calculated rate of mutation would still be 3000 times less than the spontaneous mutation rate for mammalian genomes.

Another theoretical concern regarding DNA vaccines is the potential of the foreign DNA to induce antibodies against DNA or other nuclear antigens and either induce an autoimmune state or accelerate the development of autoimmune diseases in people who are predisposed to developing these diseases [30]. To date there has been no evidence for induction of aberrant autoimmune disease in preclinical studies in animals following administration of DNA. In normal mice receiving single IM injections of plasmid DNA, there was no evidence of deterioration of renal function, as determined by serum BUN and creatinine measurements, or any abnormality in renal histology or pathology [10]. In a strain of lupus-prone mice, plasmid DNA injections did not accelerate renal dysfunction and histologic deterioration relative to control animals receiving saline placebo injections [10]. Studies of the *Pf*CSP VCL-2510 plasmid in mice and rabbits did not show production of ANA or anti-dsDNA antibodies and likewise showed no evidence of end organ pathologies [19].

The human volunteers in this Phase I study of the VCL-2510 *Pf*CSP malaria DNA vaccine were all healthy young adults with no previous exposure to malaria or malaria vaccines. In addition, the volunteers had no personal or family history of any autoimmune disease and had negative ANA and anti-dsDNA antibody titers by screening ELISA. Although studies in autoimmune-disease-prone animals have shown that DNA vaccines do not accelerate autoimmune diseases, we felt it appropriate to initially establish safety in a population of healthy human volunteers before vaccinating higher-risk populations.

The *Pf*CSP DNA vaccine was well-tolerated in all of the 20 volunteers in this study which included a 2500 μg dose group. In previous vaccine trials the highest dose of plasmid DNA that had been tested was 500 μg [20,21]. There were no severe or serious adverse events related to the *Pf*CSP DNA vaccine. Most of the adverse events were mild requiring no intervention or therapy (Table 1). The most common side effect, especially in the highest dose group, was mild pain and tenderness at the injection site, lasting approximately 48 h. Of the four moderate adverse events recorded during the trial, three did not appear to be related to the vaccine while one was determined to be possibly related (Table 1). This Phase I study was designed to look for major side-effects and therefore did not have a placebo group. However, as compared with previous malaria vaccine trials with adjuvanted recombinant proteins [31–33], the side effects from the *Pf*CSP DNA vaccine were few and mild. This is not surprising since it is the adjuvant component of most vaccines that is usually responsible for the side effects observed. The *Pf*CSP DNA vaccine is "naked" DNA and, therefore, contains no known adjuvants.

At the 12-month follow-up time point, one of the volunteers in the lowest-dose group was ANA positive by ELISA and had a borderline positive ANA titer by confirmatory IFAT that remained unchanged 3 months later. This laboratory result was considered clinically insignificant and incidental based on the following: (1) the ANA IFA titer remained unchanged from the pre-immunization baseline level of 1:40, which is considered borderline and found in up to 31.7% of the general population [34]; (2) the Helix ELISA has a false-positive rate as high as 49% even when assessed in individuals suspected of having autoimmune disease [35]; and (3) the volunteer had no detectable anti-dsDNA antibodies and no symptoms suggestive of auto-immune disease. No other volunteers had detectable ANA or anti-dsDNA antibodies for up to 12 months after vaccination with the *Pf*CSP DNA plasmid.

In this phase I study to evaluate safety and tolerability of a PfCSP DNA vaccine in healthy volunteers, we additionally showed that $CD8^+$ CTL responses [22] were induced but were unable to detect antigen-specific antibody responses. We cannot definitively explain our lack of induction of antibodies, but can speculate on a number of possibilities. Our previously reported results in *Aotus* monkeys [36] which showed that ID but not IM administration of the *Py*CSP plasmid induced antibodies, the current results (Table 3) demonstrating greater induction of antibodies in three inbred strains of mice with the *Pf*CSP plasmid administered by the ID route, as well as unpublished data, all suggest that the induction of an antibody response with DNA vaccines may be better achieved by the ID route of administration. Furthermore, studies in which mice were immunized with DNA vaccines that were designed for enhanced antigen secretion have demonstrated that antibody production is likewise enhanced (unpublished data). Previously published results with the plasmid used in this study [15] have shown that the VCL-2510 expressed product is present in the culture supernatant of transiently transfected tissue culture cells at comparatively low levels. Both the route of administration and the plasmid secretion profile may help explain the lack of a detectable antibody response in these human subjects.

In summary, in this first study of a malaria DNA vaccine we have found that IM administration of three doses of up to 2500 μg of plasmid DNA is safe, well tolerated and does not induce any obvious hematological or biochemical abnormalities or anti-dsDNA antibodies. We view this as a pilot study for a multi-plasmid vaccine designed to induce protective immunity against parasite challenge. The demonstration of safety and tolerability in healthy volunteers was the prerequisite first step in the evaluation of this new vaccine technology that by virtue of its simplicity has the capacity to profoundly influence vaccine development.

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C.HERIC

First Human Trial of a DNA-Based Vaccine for Treatment of Human Immunodeficiency Virus Type 1 Infection: Safety and Host Response

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A DNA-based vaccine containing human immunodeficiency virus type 1 (HIV-1) *env* and *rev* genes was tested for safety and host immune response in 15 asymptomatic HIV-infected patients who were not using antiviral drugs and who had $CD4^+$ lymphocyte counts of ≥500 per microliter of blood. Successive groups received three doses of vaccine (30, 100, or 300 μg) at 10-week intervals in a dose-escalation trial. Vaccine administration induced no local or systemic reactions, and no laboratory abnormalities were detected. Specifically, no patient developed anti-DNA antibody or muscle enzyme elevations. No consistent change occurred in CD4 or CD8 lymphocyte counts or in plasma HIV concentration. Antibody against gp120 increased in individual patients in the 100- and 300-μg groups. Some increases were noted in cytotoxic T lymphocyte activity against gp160-bearing targets and in lymphocyte proliferative activity. The safety and potential immunogenicity of an HIV-directed DNA-based vaccine was demonstrated, a finding that should encourage further studies.

Since its identification in 1981, the global AIDS epidemic has progressed inexorably to the point at which the cumulative total of human immunodeficiency virus (HIV) infections worldwide as of December 1996 was estimated by the Joint United Nations Programme on HIV/AIDS to be 29.4 million people, with 22.6 million currently living with the infection [1]. Since the introduction of zidovudine in 1987, significant strides have been made, using combinations of antiviral agents, in controlling the rate of HIV replication and immune deterioration in infected persons; however, the costs are great and the treatments are only partially effective. Thus, there is great interest in vaccines that might augment a host's immune response to HIV so that viral proliferation and immune damage is slowed or stopped [2–7]. It is also hoped that any immune response to a therapeutic vaccine strong enough to control established infection would also be effective in preventing initial infection in HIV-naive subjects.

While no single measure of immunity is predictive of in vivo control of viral replication, there is mounting evidence that an efficacious therapeutic vaccine against HIV-1 will need to induce both cellular and humoral immune responses to control infection. Patients mounting strong gp160-specific cytotoxic T lymphocyte (CTL) responses have shown a rapid reduction of acute viremia and antigenemia, and a high level of CTL activity has been associated with long-term survival [8, 9]. In contrast, primary viremia and antigenemia were poorly controlled in patients in whom virus-specific CTL activity was low or undetectable. High levels of antibodies, most notably neutralizing and cross-reactive neutralizing antibodies, have also been observed in the sera of long-term survivors [8] and in mothers who do not transmit HIV-1 to their babies [10–13]. More recently, it has been determined that a higher level of in vitro proliferation to p24 is associated with long-term nonprogression and in vivo control of viral replication [14]. In general, stronger and broader immune responses have been associated with delayed disease progression and are likely to have a positive impact on limiting replication, although the ability of the immune response to affect viral replication in infected persons remains controversial.

Recently, several groups have reported on the ability of DNA vaccination to lead to in vivo expression of the encoded gene products [15–17] with the concomitant development of specific cellular and humoral immune responses directed against these antigen(s) [18–22]. The endogenous production of antigen by the host cell transcriptional machinery mimics aspects of live attenuated vaccines without the associated risk of potential pathogenic replication.

DNA-based vaccination has stimulated humoral and cellular responses to HIV-1 antigens in mice [23–26] and macaques [26, 27]. More recent studies in infected chimpanzees [28] have

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Informed consent was obtained from all patients. Human experimentation guidelines of the US Department of Health and Human Services and those of the authors' institutions were followed in the conduct of this clinical research.

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shown a possible application of this strategy in HIV-1–infected persons: DNA vaccination of HIV-1–infected chimpanzees with a construct that drives expression of HIV-1 env and rev appeared well-tolerated. Importantly, immunized animals demonstrated a boost in antibody responses followed by a >1 log decrease in their virus loads [28].

The present study is an open-label phase I dose-escalation study of APL 400–003, a DNA plasmid construct containing genes that encode the env and rev proteins of HIV.

Materials and Methods

Study design. Sequential groups of 5 subjects received 3 intramuscular injections of vaccine at the same dose (30, 100, or 300 μg) at 10-week intervals. The group of subjects to receive the next escalation of dose did not begin until 2 weeks after all 5 persons in the previous dose level had received their first dose without complication. The toxic dose that would trigger study discontinuation was defined as the lowest dose at which 2 of the 5 dose-cohort patients experienced any treatment-related adverse effect greater than or equal to grade 3 (World Health Organization criteria) or the dose at which a second adverse effect of the same type occurred.

The vaccine was administered in the Clinical Research Center (CRC) of the Hospital of the University of Pennsylvania. An intravenous catheter was inserted into each subject's arm before each injection, and constant monitoring of clinical state and vital signs was carried out for 2 h after administration of the vaccine. If stable, the subject was then allowed to leave the CRC. A brief follow-up visit occurred 24 h later, and subjects returned for monitoring visits 1, 2, 4, 6, and 10 weeks later. If no adverse experiences were noted at 10 weeks, the second dose was administered, and the same 10-week cycle was repeated until the third dose at 20 weeks. Thereafter, the monitoring cycle was repeated a third time, and a final monitoring visit occurred 36 weeks after the first inoculation. In addition to the monitoring visits, patients kept a symptom log which was reviewed at each visit. Data from the monitoring visits were shared with the subjects as the study proceeded, and subjects were reminded that they were free to withdraw from participation at any time.

Inclusion and exclusion criteria. Inclusion criteria required a positive serum HIV ELISA antibody test confirmed by Western blot analysis, $CD4^+$ lymphocyte count ≥500/μL of blood, normal serum chemistries and complete blood cell count (CBC), liver enzymes ≤1.5 times the upper limits of normal, and, for women, a negative pregnancy test and commitment to the use of effective birth control for the duration of the study.

Exclusion criteria included prior receipt of any HIV vaccine, antiretroviral therapy within the preceding 3 months, presence of HIV-related symptoms, evidence of active hepatitis B or C infection or of antinuclear antibodies, history of an immunosuppressive disease (other than HIV) or medication, allergy to amide-type local anesthetics, or any acute or chronic debilitating illness unrelated to HIV infection.

Monitoring studies. Standard laboratory tests included CBC; urinalysis; serum levels of creatinine, liver enzymes, creatine kinase, and antinuclear antibodies; and erythrocyte sedimentation rate. Each subject had a chest radiograph and a cardiograph on entry and a cardiograph at week 36 (study's end). Safety was defined as lack of untoward clinical or laboratory events, with particular attention to local and systemic reactions, as well as evidence of anti-DNA antibody or muscle enzyme elevations.

Monitored HIV-related parameters included

(1) flow cytometry for $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes (performed in the Medical Center's AIDS Clinical Trials Group–approved immunology laboratory).

(2) quantitative HIV RNA polymerase chain reaction (PCR) of plasma, which was separated from cells and frozen at −70°C until shipped on dry ice for assay at LabCorp, Clinical Trials Testing Services (Research Triangle Park, NC).

(3) antibodies against HIV env, measured by ELISA done with modifications of previously described methods [10]. Briefly, recombinant gp120 based on the MN sequence (Immunodiagnostics, Bedford, MA) was resuspended in 1× PBS to a concentration of 0.5 μg/mL, and 50-μL volumes (25 ng) were incubated overnight at 4°C in 96-well plates. Next, plates were rinsed and incubated with blocking buffer for 2 h at 37°C. Serial serum dilutions were then incubated in triplicate wells for 1 h at 37°C, washed, and reincubated for 1 h with a goat anti-human immunoglobulin (Sigma, St. Louis). After being washed, the plates were color-developed and read at 450 nm. Two human HIV-1–seronegative sera were used as sample controls, and BSA-coated wells were used as negative binding controls.

(4) lymphoproliferative responses to mitogens and to HIV and tetanus toxoid antigens as determined using the method (described below) of Wang et al. (26). Briefly, peripheral blood mononuclear cells (PBMC) were separated by use of ficoll-hypaque. The PBMC were then resuspended to a concentration of 1×10^6/mL, and 100-μL aliquots were added to 96-well U-bottom microtiter plates. HIV gp120 baculovirus-produced protein (100 μL; Intracel, Issaquah, WA) was added to the wells in triplicate to final concentrations of 5.0, 0.5, and 0.05 μg/mL. The cells were incubated at 37°C in a CO_2 incubator for 3 days, and then 1 μCi of tritiated thymidine (DuPont NEN, Wilmington, DE) was added to each well and incubated overnight (12–18 h). Plates were harvested on an automatic 96-well harvester, and the amount of incorporated tritiated thymidine measured in a microbeta counter. The stimulation index (SI) was calculated by dividing the counts per minute (cpm) from cells exposed to specific stimulation by the cpm from cells incubated in media alone. To assure that cells were healthy, the nonspecific mitogen phytohemagglutinin (5 μg/mL) was added as a positive control to 3 wells. An increased response following immunization was defined as a rise in SI of ≥4 above the preimmunization value. Other baculovirus-derived HIV proteins were included in the assays as controls and consistently failed to induce responses.

(5) CTL activity against vaccinia-infected autologous Epstein-Barr virus–transformed B cell lines expressing gp160. Blood was delivered to the laboratory within 30 min of phlebotomy, and PBMC were isolated by standard ficoll-hypaque separation, washed three times with PBS, and frozen in liquid nitrogen until the time of assay. At that time, they were resuspended in RPMI containing 10% fetal calf serum, 20 U/mL interleukin-2, and 5 μg/mL phytohemagglutinin. The PBMC were cultured for ~3 weeks and then used in the CTL assay [29]. After 3 weeks' stimulation with media containing interleukin-2, the expanded T cells

were ≥85% $CD8^+$ as determined by flow cytometry. Background experiments using HIV-positive control specimens processed in parallel established that results from frozen cells were generally 10% lower than with fresh specimens. The CTL response to HIV-1 antigen in infected human subjects is generally persistent and has been detected in 40%–60% of infected persons. The response is often of sufficient magnitude that activity can be detected using freshly isolated PBMC without the usual specific in vitro stimulation necessary to detect CTL activity in other viral infections [30]. Target cells were infected with recombinant vaccinia expressing either the HIV-1 envelope gp160 (VMN462) or the irrelevant protein β-galactosidase (VSC8) (AIDS Reagent Reference Repository, Rockville, MD). These targets were washed once with serum-free medium, labeled for 2 h at room temperature with 50 μCi of ^{51}Cr (DuPont NEN), washed three times with serum-free medium, and diluted with effector cells at target-to-effector cell ratios of 1:12, 1:25, 1:50, and 1:100 in 96-well microtiter plates. To determine the spontaneous and maximum chromium release with cell lysis, three wells containing only targets were mixed with 100 μL of media or Triton X-100, respectively. After the effector and target cells were incubated at 37°C in 5% CO_2 for 5 h, 100 μL of supernatant was removed, and the amount of ^{51}Cr release was measured in a gamma counter. Specific CTL release was calculated as 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. An increased response following immunization was defined as a rise of ≥10 percentage points above the preimmunization percentage value at one or more time points. Results are presented as lysis of targets expressing HIV-1 envelope minus lysis of targets infected with wild-type vaccinia.

Plasmids. APL 400–003 is a plasmid that encodes a modified *env* gene and a *rev* gene from an $HIV\text{-}1_{MN}$ isolate. These genes are under the control of the human cytomegalovirus promoter. The plasmid contains no HIV long-terminal repeats nor the HIV-1 packaging signal. Accordingly, although it can express gene products, the plasmid cannot undergo retrovirus-directed integration, and the viral mRNA cannot become packaged into a virus particle. The plasmid is formulated with bupivacaine (an amide type anesthetic), which enhances in vivo transfection, gene expression, and immune responses as previously described [31]. The study was conducted under a Food and Drug Administration (FDA) Investigational New Drug (IND) application submitted on 18 November 1994, and it was initiated on 6 June 1995. This represents the first FDA IND for a DNA vaccine.

Measurement of HIV in semen. Semen specimens were provided by the 10 men in the 100- and 300-μg dose cohorts before their first vaccine dose and again 1 week after their third vaccine dose. Three men also provided specimens 1 week after the second dose. Specimens were brought to the CRC at ambient temperature within 4 h of ejaculation, mixed 2:1 (vol:vol) with a denaturing solution, homogenized by repeated pipetting, and aliquoted into 3.6-mL polypropylene screw-top freezer tubes (Corning Costar, Cambridge, MA). Then, the specimens were frozen at −70°C until shipped overnight on dry ice to the University of Washington (Seattle) Retrovirus Laboratory for RNA PCR quantitation. The protocol for extraction, amplification, and detection of HIV RNA from semen used a modification of the Amplicor Monitor RNA PCR (Roche Molecular Systems, Somerville, NJ) method [32], including adsorption of the RNA to silica beads [33], followed by multiple washes and ending with a resuspension of the RNA into the Amplicor sample diluent. The lower limit of detection for the assay was 400 copies/mL, and assay variability for the same specimen from day to day ranged up to 50% (Lawrence Corey, personal communication). Similar variability has been reported by others [34, 35].

Table 1. Numbers and percents of $CD4^+$ and $CD8^+$ lymphocytes and HIV plasma virus load in 15 HIV-1–infected subjects before immunization with a DNA-based vaccine containing HIV-1 *env* and *rev* genes.

	Mean	Range
$CD4^+$ lymphocytes		
No./μL of blood	705	410–960
Percent	31.0%	23%–41%
$CD8^+$ lymphocytes		
No./μL of blood	1186	515–2405
Percent	49.0%	25%–60%
Virus load,* copies/mL plasma	8694	<400–22,620

NOTE. Lymphocyte counts were determined by flow cytometry.
* Eight subjects had levels <6000 copies/mL; 1 subject had undetectable levels (<400 copies/mL).

Results

Characteristics of participants. Thirteen men and 2 women participated in the study. The median age was 36 years (range, 25–45). Twelve were white, 2 Hispanic, and 1 black. HIV acquisition categories included 13 men who had sex with men and 2 women with heterosexual exposure. Their median time since the diagnosis of HIV infection was 6 years (range, 0.5–10). Initial values represent the average of 2 determinations at 2-week intervals; 60% of repeat determinations varied by <20%, but the remainder had second values as much as 57% higher or lower than the first, a well-known phenomenon in subjects with high CD4 cell counts. Mean preimmunization flow cytometry lymphocyte values are shown on table 1. The mean $CD4^+$ lymphocyte count was 705 per microliter of blood (range, 410–960). (The 1 subject with a CD4 cell count <500/μL on the day of immunization had had >500/μL at his last screening visit.) The mean concentration of HIV in plasma (virus load) varied from undetectable (<400 copies/mL) in 1 subject to 22,620 copies/mL (mean, 8694) for the group. The mean prevaccination concentration was <6000/mL for 8 of the 15 participants.

Safety monitoring results. The immunizations were well-tolerated. No changes occurred in vital signs following injection or on follow-up visits. Four subjects (27%) noted grade 1 tenderness, lasting <1 day in 3 and 16 days in 1, following one of their injections; 3 subjects had transient nausea; 1 subject each had lethargy or night sweats after the first dose only. No significant changes occurred in CBC, serum creatinine, other blood chemistries, or urinalysis. Serum liver chemistry values

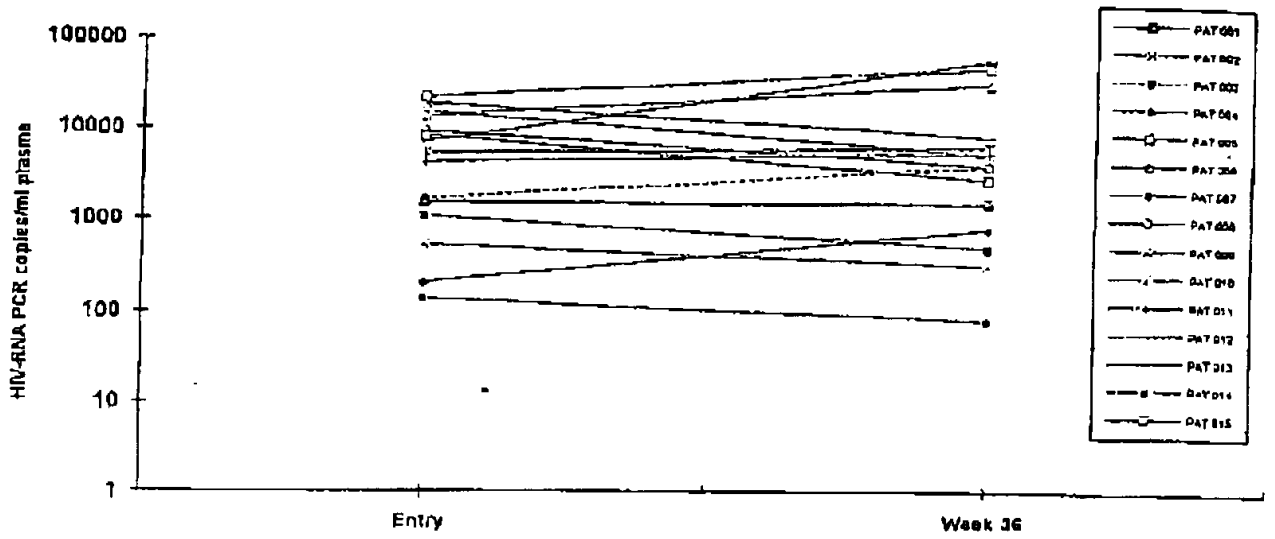

Figure 1. HIV quantitative plasma RNA polymerase chain reaction values (copies/mL of plasma) for each patient (PAT 001–015) at study entry and at 36 weeks (16 weeks after 3rd injection). Patients 1–5 received 30-μg doses, patients 6–10 received 100-μg doses, and patients 11–15 received 300-μg doses.

remained within normal range in most subjects and increased by >50% above the upper limit of normal in 3: Bilirubin increased to 1.7 mg/dL in 1 subject, and alanine aminotransferase and aspartate aminotransferase increased to 2.5 times the upper limit of normal in 2 subjects. All levels returned to normal during the study.

The erythrocyte sedimentation rate increased to 32, 37, 55, and 57 in 1 subject each but returned to normal during the study. Creatine kinase increased to twice the normal upper limit in 2 subjects and returned to normal limits while they received subsequent doses. Two subjects had random glucose determinations between 110–150 mg% on 5 occasions. One subject had persistent mild proteinuria (trace amounts to 100 mg) throughout the study. Of note, no subject developed abnormal vital signs following injection, no significant increases in antinuclear antibodies titer were observed, and anti-DNA antibody was not detected.

Plasma HIV RNA PCR copies. Figure 1 shows the plasma RNA PCR values for each subject at the time of study entry and again at 36 weeks (16 weeks after the third injection). No clear pattern of rising or falling copy number is evident. Because differences of up to 0.5 logs can be seen due to assay variability [29], we collated changes in excess of that magnitude: 2 subjects experienced a rise in plasma copy number of ≥0.5 log (from 2.32 to 2.90 and from 3.86 to 4.75); no subject experienced a decrease of ≥0.5 log, although 1 declined from 4.19 to 3.72 logs. We also looked for transient changes immediately after each injection: 7 of the 45 total injections were associated with a transient rise of ≥0.5 log, and 4 were followed by a transient fall of ≥0.5 log.

Flow cytometry results. Figure 2 shows the CD4 and CD8 values for each subject at the time of study entry and again at 36 weeks. As with plasma HIV, no clear pattern is evident. Because of inherent variability in these values in the same subject from day to day, we arbitrarily chose to regard a change of >20% from baseline as potentially significant. CD4 cell counts increased by ≥20% in 3 subjects (CD4 cell percents increased in 3) and CD4 cell counts decreased by ≥20% in 5 subjects (percents decreased by ≥20% in only 1 subject). The mean CD4 cell count for the group was 764/μL on entry and 692/μL at week 36 (compared with an average of an 80-cell loss/year in some natural-history studies). CD8 lymphocyte counts increased by ≥20% in 1 subject (CD8 cell percent increased in none) and fell by ≥20% in 4 (percent decreased in only 1). The mean CD8 cell count for the group on entry was 1199/μL, and it was 1094 at week 36.

Antibody responses. Table 2 shows the preimmunization geometric mean antibody levels against Env gene product for each subject (average of specimens obtained 2 weeks before and the day of the first injection) soon after completing the primary series of injections (week 21–24) and at week 36 (16 weeks after the third and last immunizing injection to determine the durability of any changes noted). Values for the 30-μg cohort demonstrated essentially no increase in geometric mean titer (GMT). Persons in the 100- and 300-μg cohorts showed increases of more than twice baseline level at weeks 21 or 36

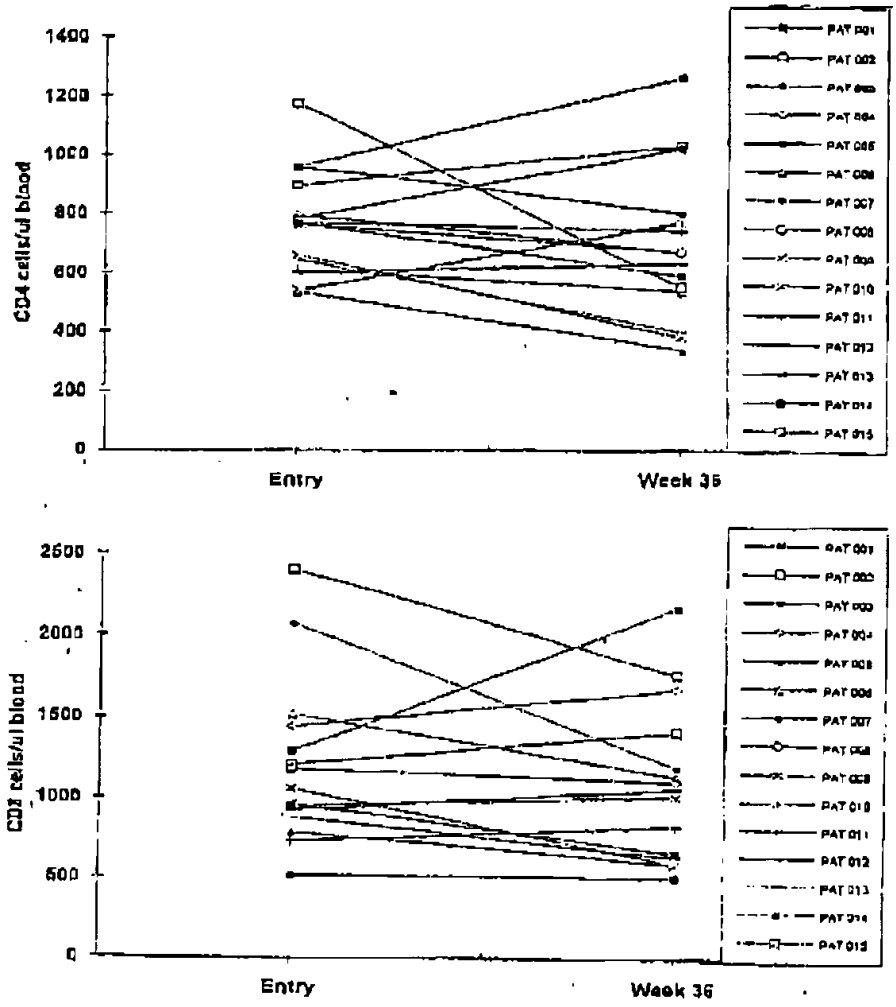

Figure 2. Absolute number of $CD4^+$ and $CD8^+$ lymphocytes/μL of blood for each patient (PAT 001–015) at study entry and at 36 weeks.

(or both). Thus, there appeared to be a threshold dose of vaccine above which an antibody response could be anticipated.

CTL responses. Because we were not able to immortalize lymphocytes from all participants, specimen availability permitted assay in 10 of the 15 vaccine recipients for specific CTL responses against HIV-1 gp160–expressing targets. Table 3 shows the CTL activity for subjects in each dose cohort, expressed as percent specific lysis, at the same monitoring times as sampled for antibody above. No increase in CTL activity was seen in the group that received 30-μg vaccine doses, whereas 2 of the 3 subjects in the 100-μg group showed increases of 10 percentage points or more in specific CTL. Perhaps related to high preimmunization CTL activity is the fact that none of the 3 subjects in the 300-μg group had increased CTL activity after vaccination. The relationship between vaccination and the rises in individual patient's CTL activity is uncertain.

Lymphocyte proliferative responses. Specimen availability allowed for assay of lymphoproliferative responses directed against HIV antigen and against phytohemagglutinin for cells from 3 subjects at each dose level. Table 4 shows responses (expressed as SI) to gp120 of preimmunization lymphocytes and of lymphocytes 21 and 36 weeks after the first dose. Increased proliferative responses (SI $\geq$4 above baseline) were noted at all vaccine concentrations, without a clear dose response.

Quantitative HIV RNA PCR of semen. Semen specimens from the 10 men who received the 100- and 300-μg vaccine doses were tested. Five had levels of HIV RNA below the level of detection (<400 copies/mL of plasma) before vaccination.

Table 2. Geometric mean titers of serum antibody against HIV-1_{MN} gp120 in 15 HIV-1–infected patients immunized with various doses of a DNA-based vaccine containing HIV-1 *env* and *rev* genes.

Vaccine dose, patient no.	Geometric mean titer		
	Preimmunization	Week 21	Week 36
30 μg			
001	655,000 ± 262,000	131,000	262,000
002	524,000 ± 45,000	1,049,000	1,049,000
003	1,049,000 ± 0	1,049,000	1,049,000
004	492,000 ± 406,000	262,000	262,000
005	524,288 ± 371,000	524,000	262,000
100 μg			
006	109,000 ± 38,000	131,000	66,000[a]
007	295,000 ± 165,000	1,049,000[b]	524,000
008	393,000 ± 151,000	1,049,000	1,049,000
009	393,000 ± 131,000	262,000	524,000
010	164,000 ± 66,000	524,000	1,049,000
300 μg			
011	38,000 ± 25,000	131,000[b]	524,000
012	197,000 ± 197,000	524,000	1,049,000
013	98,000 ± 98,000	131,000	131,000
014	786,000 ± 303,000	1,049,000	1,049,000
015	481,000 ± 486,000	524,000	1,049,000[b]

NOTE. Titers were determined by using highest dilution of antisera with optical density (OD) at 450 nm that was elevated compared with level of reactivity in normal human sera. End-point titers were determined by extrapolating to titer that was nearest to 3 SD above OD of normal human sera at its lowest dilution.

[a] Determined at weeks [a]30, [b]25, [c]26, and [d]34.

Table 3. Specific lysis of HIV-1 gp160-expressing targets at an effector-to-target cell ratio of 50:1 for 10 of 15 HIV-1–infected subjects vaccinated with various doses of a DNA-based vaccine containing HIV-1 *env* and *rev* genes.

Vaccine dose, patient no.	% specific lysis		
	Week 0	Week 21	Week 36
30 μg			
002	29.2	0	0
003	16.6	12.7	14.9
004	16.3	13.7	19.9
005	5.3	0	0
Mean	16.9	6.4	8.7
100 μg			
007	2.1	0.7	1.3
009[a]	14.9	26.7	18.8
010	10.3	19.6	21.5
Mean	9.2	15.7	13.9
300 μg			
011[a]	48.2	9.9	25.7
012	10.7	8.3	0
014	34.8	32.9	29.5
Mean	31.2	17.1	18.4

NOTE. Target infected with VSCB (recombinant vaccinia expressing β-galactose) was used to assess background lysis of vaccinia-infected cells. Spontaneous release was <30% of maximum.

[a] Spontaneous release ~32%–43% of maximum.

Table 4. Proliferative responses (expressed as stimulation index [SI]) of lymphocytes against gp120 (5 μg/mL) in 9 of 15 HIV-1–infected subjects immunized with various doses of a DNA-based vaccine containing HIV-1 *env* and *rev* genes.

Vaccine dose, patient no.	SI		
	Preimmunization	Week 21	Week 36
30 μg			
002	2.2	8.8	4.7
004	17.0	18.9[a]	3.3
005	8.4	8.4	20.1
Mean	9.2	12.0	9.3
100 μg			
006	16.1	28.4	26.7
007	3.0	1.7	1.1
008	8.2	8.1	14.6
Mean	9.1	12.7	14.1
300 μg			
011	5.4	15.4	11.5
014	9.5	14.5	8.5
015	7.4	10.1	12.1
Mean	7.4	13.3	10.7

NOTE. SIs were calculated by dividing amount of proliferation to gp120 by proliferation of media alone. Phytohemagglutinin was used as positive control and to assess health of peripheral blood mononuclear cells. gp120 was derived from HIV-1_{MN}. Preimmune data are averages for 2 preinoculation time points.

[a] Week 11.

and 5 had counts of 751, 790, 900, 957, and 7003 copies/mL. Specimens obtained during week 21, 1 week after the third immunization, from the 5 men who had detectable virus in semen before vaccination and 1 whose level was <400 were assayed. Two specimens from the 5 men who originally had detectable HIV RNA had levels below the level of detection on week 21, and the other 3 men had counts 21%, 41%, and 109% of their preimmunization values. At 21 weeks of study, virus was still undetectable in semen from the subject whose original specimen had undetectable levels of virus.

Discussion

The role of the immune response in controlling viral replication in vivo is under investigation and is an important subject of debate. In the majority of cases, the natural immune response generated against HIV-1 does not prevent the eventual onset of AIDS. However a minority of patients, termed nonprogressors, tend to share a similar immunologic phenotype that includes strong antiviral cellular as well as humoral immune responses [36]. Patients mounting strong gp160-specific CTL responses have shown a rapid reduction of acute viremia and antigenemia, and a high level of CTL activity has been associated with long-term survival [8, 9]. However, primary viremia and antigenemia were poorly controlled in patients in whom virus-specific CTL activity was low or undetectable. Further-

more, a definite CTL response has been observed in Gambian sex workers who remain seronegative as well as PCR-negative for HIV-1 despite repeated exposures [37, 38]. High levels of neutralizing and cross-reactive neutralizing antibodies have also been observed in the serum of long-term survivors [8]. Other conceptually related studies demonstrated that stronger serologic responses are present in mothers who do not transmit HIV-1 to their babies [10–13].

Although this observation is controversial, stronger and broader immune responses have been associated with delayed disease progression, and are believed to limit viral replication, although the ability of the immune response to affect viral replication in infected individuals remains unsubstantiated [39]. Overall, it is thought that a useful goal for immune therapy of HIV would be to raise and broaden antigen-specific anti-HIV cellular and humoral responses.

Recent efforts have demonstrated the safety and immunogenicity in humans of several recombinant protein products, including recombinant gp160 [2, 3], LAI gp120 [4], and MN gp120 [5]. In addition, there is some evidence to suggest that vaccination with recombinant envelope and vaccinia preparations used as immunotherapy can expand anti–HIV-1 immune responses [6, 7, 39–42]. Although these immunogens did not affect HIV virus load, some impact was noted on antiviral cellular and humoral immune responses. Accordingly, examination of an immunogen that can induce cellular immunity is important for developing immune therapy for HIV.

DNA vaccines represent a novel application of gene therapy, which has as one of its strengths the ability to induce a cellular immune response. Specifically in the case of HIV, both immunotherapeutic as well prophylactic applications of DNA vaccines have shown the ability to impact viral replication in animal models [18, 23–27].

DNA vaccines have not been evaluated previously in humans. The primary objective of this study was to determine the safety of an HIV DNA vaccine in HIV-infected subjects. We found that the intramuscular administration of vaccine containing the *env* and *rev* genes of HIV, given as a series of 3 injections at 10-week intervals, using doses of 30, 100, or 300 μg, was safe (i.e., without significant clinical or laboratory complication) in 15 subjects. No local injection reactions were observable, and the 4 subjective reports of local tenderness were mild and not recurrent. Subjects experiencing nausea admitted to being anxious about receiving their injection. One subject's observation of a "scratchy throat" was coincident with an upper respiratory infection. Liver chemistry elevations were mild, and one episode was related to an acute attack of hepatitis A in the subject. Sedimentation rates rose transiently in 4 subjects and returned to normal while the subjects remained in the study. Of note, the subjects lacked antinuclear antibody formation, muscle enzyme elevation, and changes in blood pressure and pulse. Thus, the short-term negative effects of the vaccine were negligible, and no participant withdrew because of adverse effects.

A secondary objective of the study was to monitor for changes in the course of the subjects' HIV infection, utilizing the surrogate markers of $CD4^+$ lymphocyte counts and plasma HIV RNA PCR copies. The absolute numbers and percents of $CD4^+$ and $CD8^+$ lymphocytes varied but without obvious pattern. Some subjects demonstrated both a 20% increase and a similar decrease at different times during the study. The plasma RNA PCR copy number has been reported by some investigators to increase after receipt of other vaccines, such as influenza and tetanus, although study results have been mixed [43–47]. Numbers in our vaccine recipients failed to show a clear trend.

When values at the end of study (week 36) were compared with the patients' starting values, significant increases in virus load were only detected in 2 of 15 subjects. Transient increases of ≥0.5 logs were noted after 7 of the 45 injections, whereas transient decreases of the same degree occurred after 4 injections. The absence of sustained increases in plasma virus load further supports the benign nature of this approach. On balance, there was no clear trend to indicate that receiving the vaccine either was detrimental or beneficial to the course of the two surrogate markers of HIV infection. In addition, attempts to quantitate HIV RNA in semen by PCR in 10 men revealed detectable virus in 5; however, after vaccination, the values were lower or undetectable in 4. If changes in virus load in this compartment were observed in an adequately powered study, they could indicate a potential use for such a vaccine to reduce HIV transmission.

Our other secondary objective was to determine whether administration of an HIV DNA vaccine to subjects already infected with the virus would or would not induce changes in measurable immune responses. Both antibody- and cell-mediated immune responses were studied. We compared antibody GMTs against gp120 as well as specific CTL responses and T lymphocyte proliferation from the subjects measured at three times: immediately before initiating the 3-dose series of injections, immediately after the third dose (week 21), and 4 months later (week 36). Although the sample sizes were too small to make conclusions, there were interesting differences observed based on dose group. For example, there was a lack of apparent boosting in antibody titers in the 30-μg dose group, but subjects in the 100- and 300-μg groups displayed rises in GMTs by 36 weeks. Examination of CTL responses showed that 0 of 4 patients in the 30-μg group had a ≥10 percentage point increase in their CTL level compared with prevaccination levels. However, 2 of the 3 patients in the 100-μg group had an increase of ≥10 percentage points in CTL responses at one or more time points during follow-up. The lack of an increase in CTL activity in the 300-μg group may have resulted from high levels of activity present before vaccination in 2 of 3 subjects. However, the inherent variability of the CTL assay makes it unwise to draw any firm conclusion and indicates that further studies will be required to resolve this issue. Lymphocyte proliferative responses did not show a dose-dependent response: The SI increased by ≥4 in 2 of 3 patients in the 30-

ug dose group, 2 of 3 patients in the 100-ug dose group, and 3 of 3 in the 300-ug dose group at one or more times during follow-up. It is interesting that there was a response to the vaccine at all in the context of ongoing virus infection.

The present study demonstrates the safety and tolerability of a DNA-based vaccine as potential immunotherapy in HIV-1–infected subjects. The results, however, do not allow us to draw conclusions regarding the effect of these immunogens on viral replication. Our protocol was designed conservatively, with a 10-week interdose interval based on safety priorities rather than choosing a shorter dosing frequency shown to be effective in prior primate studies [28, 48].

Additional studies examining different dosing and immunization regimes as well as including additional targets as immunogens are likely to improve any effect of this approach on HIV replication. In this regard, human testing of a second plasmid construct containing HIV-1 *gag* and *pol* genes as well as simultaneous administration of *env/rev* and *gag/pol* constructs is under way. Recently, our group has reported on the use of molecular adjuvants to potentiate DNA vaccine immunogens [49, 50]. We demonstrated that such an engineered immunogen, containing both pathogen-specific genes and cDNA cassettes promoting a specific immune response, can increase the specificity and magnitude of the resulting immune response in animal systems. Such an approach might improve the immunogenicity and impact of a therapeutic vaccine. Finally, the present study was initiated prior to the recent reports of the success of highly active antiretroviral therapy (HAART) in suppressing plasma HIV concentration and increasing CD4 lymphocyte counts [51–54]. To determine whether control of virus production will allow recipients to respond more vigorously to the vaccine, leading in turn to enhanced immune control of the infection, we have initiated a study of HIV DNA vaccine immunotherapy in patients whose virus burdens are maximally suppressed by HAART.

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